Agriculture and Agri-Food Canada | Agriculture et Agroalimentaire Canada

ONTARIO RESEARCH ENHANCEMENT PROGRAM (O R E P)

Final Report

MICROPROPAGATION OF NORTH AMERICAN GINSENG

REPORT No. OREP - 1999/21

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Canada

103-02942/

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September 2001

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FORWARD

The Ontario Research Enhancement Program (OREP) is a $4-million two-year federal research initiative that is administered by the Research Branch of Agriculture and Agri-Food Canada (AAFC), with input from the agriculture and agri-food sector, universities and the province. Research focuses on two areas identified by the sector as:

1. Priorities responding to consumer demand for higher quality products; and
2. Ensuring crop-production management systems are environmentally sustainable.

Biotechnology is one of the areas to be explored. Ontario's agricultural production is based primarily on growing diversified crops using intensive production practices, but long-term viability is linked to the development of sustainable crop production management systems.

There are also opportunities to enhance the economic contribution of Ontario's agriculture and
agri-food sector by adding value to the diversified primary commodities produced in the province. The Program is expected to be of particular interest to the corn, soybean, greenhouse, fruit and vegetable sectors and the emphasis will be on projects focussing on food quality improvement and sustainable crop production management such as:

1. The development of value-added animal and crop food products and ingredients that meet domestic and export market demands, with a particular emphasis on quality issues such as processing, packaging, shelf life, new product and ingredient development, high-value alternative uses, probiotics and functional foods;
2. Biotechnologies to reduce environmental pressures related to crop protection practices; and,
3. Integrated crop protection and production practices for both field and greenhouse crops.

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EXECUTIVE SUMMARY

North American ginseng (*Panax quinquefolius)* has been harvested in North America as an export crop to Asia since the 1700's. It is highly valued for its medicinal and herbal use and complements Korean ginseng (*Panax ginseng*) in traditional Eastern medical practice. In the last century, cultivated ginseng in North America has been a growing export crop. In recent years, commercial production of ginseng has expanded rapidly and Canada is now the world's largest producer of *P. quinquefolius*. In Ontario, ginseng is the fifth most important cash crop after soybean, corn, tobacco, and wheat. The potential yield of the cultivated ginseng crop is limited by several factors including disease, soil nutritional status, competition from weeds, and poor seed germination. The relatively high cost of ginseng seed, weed and pest control and bed maintenance along with the lack of proven ginseng cultivars make efficient establishment of plants in cultivation beds a critical step in the production of this crop.

A sizable transplant industry has been developed for crops such as tobacco and tomato in Southern Ontario. For ginseng, micropropagated transplants could be an effective approach to boost the expansion, efficiency and profitability of the ginseng industry. Tissue cultures are started with very small pieces of plant tissue and produce small true-to-type clonal propagules, which require a relatively small amount of space to maintain and increase plants. The nature of the propagule that results from micropropagation is a "micro" plant; it is an ideal product for field delivery as a transplant in the form of a transplant plug. Propagation of the transplant plugs is carried out under aseptic conditions which allows cultures to be treated to cure the cells of viral and bacterial contaminants producing plants which can be certified as pathogen-free. In a microprpagation/transplant system, environmental and nutritional factors can be closely controlled so that cultures can be propagated year round. The rate of *in vitro* propagation is much greater than traditional seed propagation and will allow a more rapid deployment of selected, improved or new genetic lines. For ginseng, micropropagation obviates the approximate 20-month dormancy period needed for normal seed germination. This gives a substantial time saving of two growing seasons for each cultivation or breeding cycle. An additional advantage of true-to-type (micro)propagation is the ability to rapidly multiply specific and unique plant types such as plants with specific ginsenoside profiles or plants with enhanced disease resistance.

In the work described here, a micropropagation system for *P. quinquefolius*, based on an 9-stage protocol involving somatic embryogenesis and multiple shoot formation, has been developed. Somatic embryos can be induced from seed-derived tissue of mid-mature zygotic embryos cultured on growth regulator-free, high sucrose, Murashige and Skoog (MS) medium. After seven weeks

in culture, somatic embryos form on up to 90% of the explants at up to 20 embryo propagules per explant. Germination of somatic embryos and subsequent shoot development and elongation occurs after an additional four to eight weeks on MS medium supplemented with the plant growth regulator gibberellic acid. Somatic embryo induction and shoot regeneration from cotyledon explants does not appear to be genotype dependent, although the response of individual explants (cotyledons, petioles, leaves and roots) is variable. Rooted plantlets can be obtained within 120 days from the start of culture. A similar protocol can be used to produce propagules from petiole sections of established plants. Plants can be induced to form a dormant bud that, after a simulated overwintering period of 90-120 days, form normal-looking ginseng plants. Tissue culture derived plants have been planted out in prepared beds along with seed-derived control plants. An added advantage of the overwintering treatment of tissue-culture derived plants is their ability to undergo reduced flowering; an advantage for accelerating a germplasm multiplication or a breeding program. The efficiency of the first five of nine stages of the protocol is high (90-95%) and is an effective method for genotype multiplication. The efficiency of the latter stages of the protocol involving shoot development, rooting and acclimatization to green house/ field conditions ranges from 25-65% and requires improvement. Although root production is at the 50% stage, there is a need to improve root quality as the field establishment efficiency of the last stage at 11% and over wintering at 3% suggests that root quality of micropropagated plants is a limiting (but solvable) factor for use in breeding or germplasm development programs and for commercial application.

This work is the first complete summary on the performance of tissue culture-derived plants of *P. quinquefolius* from introduction into culture to acclimatization and introduction into the field with an evaluation of field performance including overwintering.

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ABSTRACT

A laboratory-scale micropropagation system for *P. quinquefolius*, based on an nine-stage protocol involving somatic embryogenesis and multiple shoot formation, has been developed. Somatic embryos can be induced from seed-derived cotyledon explants of mid-mature zygotic embryos cultured on growth regulator-free, high sucrose, Murashige and Skoog (MS) medium. After seven weeks in culture, somatic embryos form on up to 90% of the explants. Germination of somatic embryos and subsequent shoot elongation occurs after an additional four to eight weeks on MS medium supplemented with gibberellic acid. Somatic embryo induction and shoot regeneration from cotyledon explants does not appear to be genotype dependent, although the response of individual explants is variable. Rooted plantlets can be obtained within 120 days from the start of culture. Plants can be induced to form a dormant bud that, after a simulated overwintering, form normal-looking ginseng plants. This work is the first complete summary on the performance of tissue culture-derived plants of *P. quinquefolius* from introduction into culture to acclimatization and introduction into the field with an evaluation of field performance including overwintering.

INTRODUCTION

Ginseng: A medicinal herb

North American ginseng (*Panax quinquefolius* L.) is a native herb of Canada that is highly valued in Eastern medical practice for its "cooling effect", for the treatment of *Qi-deficient* conditions and its effect on the cardiovascular system. In Western culture, it is viewed as a health tonic and is used to increase energy and reduce stress levels. The therapeutic effects of this herb are attributed to a group of saponins, complex carbohydrates combined with either alcohol or phenol, that are collectively known as ginsenosides (see Bergner, 1996 for an overview). Ginsenosides are present throughout ginseng plants but levels are highest in the root (Ma et al., 1995). *P. quinquefolius* is considered to be distinct and complementary to Korean ginseng (*P. ginseng* C.A. Meyer) because of differences in ginsenoside content and levels. Ginseng for use in eastern medicine is also judged for root shape as an important aspect of plant worth apart from chemical factors. While *P. ginseng* and *P. quinquefolius* are the most widely commercially available ginseng plants, a number of other species (shown in Table 1) are cultivated as medicinal herbs, mostly in China.

While most of the ginseng produced in Canada is exported to China, the increasing world-wide use of ginseng in "value added" products such as tea and snack foods, points to a future expansion of the ginseng market and Canadian acreage.

Cultivation of Ginseng

Market Value

Currently, Southern Ontario is the largest producer of *P. quinquefolius* in the world. In 1998, 3.6 million pounds of ginseng root was harvested in Southern Ontario compared to 1.7 and 1.5 million pounds harvested in Southern British Columbia and Wisconsin, respectively. Commercial production of ginseng has dramatically increased in this decade; in 1997 it had a farm gate value of over $50 million compared to only about $3 million in 1980. With yields of up to 4000 pounds of dried root per acre and wholesale prices in the $17 - $25 range, ginseng has a potential farm gate value of $100,000 per acre. Prices for the 2000 and 2001 crop are in the $22 range and yields appear to have been in the 2800 lb/ acre range Recent downward pressure on prices is thought to be due to the rapid increase in supply of dried root, slow short-term growth in the primary market (Asia), currency devaluation in the Asian market countries, a tightening of credit, competition from other suppliers and changing import channels. In Ontario, ginseng is the fifth most important cash crop after soybean, corn, tobacco and wheat.

Table 1. Ginseng species

Botanical name	Common name(s)
P. ginseng C. A. Meyer	Korean, Asian, or Oriental ginseng
P. japonicus C. A. Meyer	Bamboo, Japanese, or Chikusetsuninjen ginseng
P. major Ting	Big leaf sanchi or pearl ginseng
P. notoginseng (Burkill) F. H. Chen	Chinese Sanchi or Tienchi ginseng
P. ameiensis J. Wen	
P. pseudoginseng Wallich	Himalayan or Chinese ginseng
P. quinquefolium L.*	American, Canadian or North American ginseng
P. sinensis J. Wen	
P. stipuleantatus H. T. Tsai & K. M. Feng	Pingbiann Sanqi
P. trifolius L.*	Dwarf ginseng or ground nut
P. wangianus Sun	
P. zingiberensis C. Y. Wu & K. M. Feng	Jiangzhuang Sanqi or ginger shaped ginseng
Angelica sinensis	Woman's ginseng
Eleutherococcus senticosus	Siberian ginseng
Withana somnifera (L.) Dunal	Indian ginseng
Pfaffia panaculata	Brazilian ginseng

[a]based on Wen and Zimmer, 1996; Li, 1995. **North American species are indicated by ***

Cost of Production

While the potential farm gate value of cultivated ginseng has been attractive, the investment costs are high because of several factors. First, because ginseng is a woodland herb that grows in well-drained soil with a high organic matter content, its cultivation requires expensive climate modification. In production fields, shade structures that block 70-80% of the sunlight cost between $16, 000 and $21, 000 per acre. Bed preparation and maintenance costs are also high because ginseng is grown in raised beds with a layer of mulch to provide proper drainage and insulation. Second, the cost of disease and pest control (~$4000 per acre) and seed (up to $5000 when seed is limiting) are high. Ginseng is susceptible to a number of fungal pathogens many of which affect seed and root quality. Ginseng is a slow growing perennial with a three to five-year reproductive cycle. The seeds are susceptible to disease and stand establishment is inconsistent in part because of poor seed germination. Finally, the commercially important part of the plant, the root, is not usually large enough for commercial harvest until the third to fourth growing season. Roots can be harvested earlier but have considerably lower market value.

Anticipated benefits to the Industry

One way to reduce the high input cost and to increase profitability of the Industry is to develop ginseng cultivars with desirable traits such as disease resistance and superior root shape. Currently, there are no registered cultivars of *P. quinquefolius* and DNA fingerprinting studies have demonstrated that this species is genetically diverse (Bai et al., 1997). Since traditional breeding methods are slowed by the long reproductive cycle of this species, micropropagation technology is an attractive alternative for creating a stock of superior germplasm. Using micropropagation techniques, ginseng plants with specific ginsenoside profiles or with other genetically determined traits such as disease resistance can be selected and cloned rapidly. In conjunction with DNA fingerprinting, a micropropagation system for *P. quinquefolius*, could lead to the development of recognized ginseng cultivars with quality assurance.

In addition, development of a micropropagation/transplant system for North American could have the following potential benefits:

1. Decreased input cost due to a decreased cost of planting. Although an individual plantlet may be more expensive than a seed, only about 10-30 percent of the planting units are required at the time of harvest.

2. A consistent and predictable stand establishment with well-controlled plantlet density and uniform establishment will be possible.

3. The acclimation procedure for tissue culture-derived plants, results in the saving of two years in each three-year breeding cycle; thus, the breeding program and introgression of new germplasm would be dramatically accelerated.

4. The micropropagation/transplant procedure results in the production of a two-year-equivalent plant in the first year. The crop is sold on the basis of weight and quality; the gain of one year per three- to four-year growing season would be a substantial economic benefit

5. An *in vitro* genetic conservation system could be developed to aid the breeding program.

6. Tissue culture-derived plants could be marketed as pathogen-free when virus and bacterial indexing was performed.

Micropropagation

Research on the tissue culture of Korean ginseng has been ongoing for thirty years with reports on callus cultures, *in vitro* conservation, protoplast culture, shoot organogenesis, somatic embryogenesis and *Agrobacterium*-mediated genetic transformation (Arya, et al., 1991; Butenko et al., 1968; Chang and Hsing, 1980; Choi, 1988; Furuya et al., 1986; Lee et al., 1995; Shoyama et al., 1995; Tirajoh and Punja, 1995; Yang and Choi, 2000 and citations). Large scale culture of ginseng has been used in Russia (Zhuravlev and Muzarok, 1995) and Korea. There is no evidence that any of these techniques have been used on a commercial scale in North America or with North American ginseng. There are only a few reports on the *in vitro* cultivation of North American ginseng; these documented cases report on the formation of somatic embryos on callus cultures of embryonic or leaf explants (Jhang et al., 1974; Li and Guo, 1990; Wang, 1990; Tirajoh and Punja, 1995). Appendix 1 outlines the various protocols, critical growth medium components and regeneration efficiencies that have been reported in the literature.

There are three developmental pathways that can lead to production of new clonal propagues through the use of tissue culture techniques; development of pre-existing meristems, shoot and root organogenesis, and somatic embryogenesis. In the first case, *development of pre-existing meristems*, the isolation of a segment of plant tissue from the donor plant onto a synthetic growth

medium results in the release form growth inhibition of preformed meristematic regions and the development of new plants in culture. An example of this approach is the use of stem node sections which contain undeveloped axillary shoots that can be induced to elongate after separation form the mother plant. The elongated shoots can be induced to root in culture or after removal from culture and placement in a mist bed. In induction of *shoot organogenesis* on plant segments (typically leaf sections) placed on synthetic growth media such as the widely used Murashige and Skoog (1962) in the basis of much of the ornamental micropropagation industry. The manipulation of two classes of plant growth regulators supplied in the synthetic growth medium results in the formation of new meristematic regions which, under controlled culture conditions, will develop into well formed shoots. As in the case of the development of pre-existing meristems, a second step to induce root formation is required. *Somatic embryogenesis* is a form of cloning in which embryos are formed asexually, directly from vegetative tissue. This is the basis for the concept of synthetic seed (Brown et al., 1995). Somatic embryos, or clonal propagules, develop a propagule with shoot and root meristems that is similar in structure to a seed or sexual embryo. Although synthetic seed has not yet been used extensively commercially, it is viewed as the next generation of technology and has been successfully applied in the forestry and plantation plant Industry. This technology developed with alfalfa (McKersie and Brown, 1997) has been successfully transferred to canola and wheat at the Southern Prop Protection and Food Research Centre.

The micropropagation protocol for *P. quinquefolius* begins with the induction of somatic embryos from seed-derived cotyledon explants. Cotyledons were chosen as the explant source based on observations that cotyledon explants from zygotic embryos of *P. ginseng* are highly embryogenic (Choi et al., 1999; also see Appendix 1). Somatic embryos can only be induced in *P. ginseng* from cotyledons when these are excised from the embryonal axis (Choi and Soh 1995a). When agar blocks containing exudate from radicles or plumules were placed at the base of cotyledon segments, somatic embryogenesis was reduced. Furthermore, somatic embryogenesis was completely suppressed when cotyledon segments were cultured in direct contact with excised radicles or plumule tips. This indicates that the embryonic axis produces a substance that suppresses somatic embryogenesis. It is also suggest that wounding at the basal portion of the cotyledon segments, upon excision, causes the polar accumulation of a hormone or other substance that stimulates somatic embryogenesis on the cotyledon.

To induce somatic embryogenesis, it is usually necessary to culture plant tissue on medium containing plant growth regulators, in particular, auxins (see Appendix 1). However, this method of induction is usually accomplished indirectly, via callus formation, and poses the potential problem of somaclonal variation. Somaclonal variation is a non-specific but heritable induction of variation in the plant propagules resulting in unwanted plant types. Somatic embryogenesis can also occur in the absence of plant growth regulators, through chemical stress such as high levels of sucrose,

salts, or heavy metals. Somatic embryos can be induced directly on cotyledon explants from zygotic embryos of *P. ginseng*, without an intervening callus stage, when explants are cultured on medium containing excess macrosalts (Choi et al., 1998a) or excess sucrose (Choi et al., 1998b). Somatic embryos can also be induced from multiple shoots segments of *P. ginseng* when the segments are cultured on growth regulator-free medium containing high concentrations of sugars (Asaka et al., 1994) or in the presence of high temperatures (Asaka et al., 1993). Cotyledon explants cultured on medium containing no growth regulators produces higher numbers of somatic embryos than medium supplemented with auxin (2,4-dichlorophenoxyacetic acid or indole-3-acetic acid) or cytokinin (benzyladenine or kinetin) (Choi and Soh 1995b).

In the nine-stage micropropagation protocol for *P. quinquefolius* described below, somatic embryos are induced directly on cotyledon explants cultured on growth regulator-free medium containing excess sucrose. The same approach can be used with leaf petiole and root tissue of P. *quinquefolius* but the efficiency is lower at present; thus, cotyledon explants were used to develop the system. Subsequent shoot and root development involves the addition of growth regulators to the culture medium. This report summarizes these nine stages from introduction into culture to the performance of tissue culture-derived plants in the field, describes the optimization of the stages and identifies rate limiting factors for the technology.

OBJECTIVES

The goal is to develop and alternative delivery system for ginseng - a micropropagation/transplant system. There are three main objectives addressed below:

1. Establish a medium-scale micropropagation system to rapidly multiply selected ginseng genotypes.

2. Develop a transplant plug delivery system for micropropagated plants.

3. Evaluate the efficacy of micropropagated transplants as a delivery system for ginseng.

The focus of this research work was objective 1, to establish a medium-scale micropropagation system to rapidly multiply selected ginseng genotypes.

MATERIALS AND METHODS

Introduction into culture and culture conditions

Partially stratified seeds were obtained from 3-5 year old *P. quinquefolius* plants grown in field plots at the Delhi Field Station site of the Southern Crop Protection and Food Research Centre in Delhi, Ontario. The seed coats were removed and the naked seeds were surface sterilized by immersing in a 0.1 % Benolate (fungicide) solution for 15 minutes, 70% ethanol for 1 minute and soaking in a 20% dilution of household bleach containing a few drops of Tween 80 for 15 minutes. The sterilized seeds were rinsed three times with sterile distilled water and the zygotic embryos were excised aseptically. The same sterilization protocol was used for donor ginseng seedlings grown from stratefied seed in a growth chamber. All other tissue was obtained from *in vitro*-grown seedlings. Unless otherwise indicated, the basal medium used in all experiments was MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 2.4 g l^{-1} Gelrite. The pH of the medium was adjusted to 5.8 prior to autoclaving at 120^{0}C for 20 minutes. Cultures were maintained at 24°C with a 16-hour photoperiod of fluorescent light with an irradiance of 30-50 µmol m^{-2} s^{-1}.

Somatic embryogenesis

Effect of sucrose on embryo induction

Cotyledons of mid-mature zygotic embryos (see above section) were excised and placed abaxial side down on MS medium containing 40, 50, 60, or 70 g l^{-1} sucrose. Explants were transferred to fresh media after the first week, and every two weeks thereafter until most explants had mature somatic embryos. In the first set of experiments there were 7-8 explants per plate with at least 3 replicates per treatment and in the second set of experiments there were 9-11 explants per plate with at least 5 replicates per treatment. The number of explants producing embryos were recorded during the 2nd, 4th, 7th, and 10th week of culture. Data for week 7 are plotted as the mean percentage of explants producing embryos. Variability is represented as the standard deviation between replicates in each treatment after arcSIN transformation. At week 10, the number of embryos per explant was recorded and the data was plotted as the average number of embryos per explant for each treatment. The remaining zygotic embryos were used in additional experiments that assessed the effect of light on somatic embryo induction from cotyledon explants cultured on the same sucrose range as outlined above. The number of explants with somatic embryos were recorded at weeks 4, 8 and 10. For all experiments, general observations were made on the developmental stage, morphology, location of somatic embryos and presence of secondary embryogenesis.

Effect of abscisic acid (ABA) on somatic embryo quality

The effect of ABA on somatic embryo production and maturation was investigated for cotyledon explants. After 3 weeks of pre-culture on MS medium containing 40 g l^{-1} sucrose, cotyledon explants were transferred to the same basal medium containing 0, 0.25, 2.5, or 25 mg l^{-1} *(±) cis/trans* ABA. The experiment contained 10 replicates with at least 8 explants per replicate. Observations on the number of somatic embryos per explant, as well as the developmental stages of the embryos and occurrence of secondary embryogenesis, were made at weeks 4, 5 and 6. Cotyledon pairs were split up so that each cotyledon of a given pair was on a different treatment.

Effect of plasmolysis pretreatment on embryo induction

I. Cotyledon explants were cultured abaxial side down on MS medium containing 1.0 M (342 g l^{-1}) sucrose for 24, 72, and 120 hours and then transferred to MS with 30 g l^{-1} sucrose (controls were plated directly on MS). There were 8-10 explants per plate with at least 8 replicates for each

pretreatment time. The cotyledons were subcultured every two weeks and observations on somatic embryogenesis were made at weeks 4 and 7.

II. In a second set of plasmolysis experiments, sterilized tissues (leaf and petiole/stem) from eight 1-month-old seedlings were pretreated in an aqueous solution of 1.0 M sucrose for 24, 72, 120, and 168 hours. There were eight explants of each tissue type for each pretreatment time and the experiment contained two replicates. Petioles/stems and leaves from each seedling were cut so that there were approximately eight equal sized pieces for each tissue type. Therefore, for each seedling there was a piece of petiole/stem and leaf in each treatment for each replicate. The explants were put into 125 ml flasks containing 40 ml of 1.0 M sucrose solution and shaken at 125 rpm. After the appropriate length of pretreatment, the explants were deplasmolysed by adding an equal volume of 0.1 M sucrose to the culture flasks and swirling for 5 minutes, and then transferring to new flasks, containing 40 ml of 0.1 M sucrose, and swirling for another 5 minutes. Following deplasmolysis, the explants were transferred to plates containing semi-solid MS with 0.1M sucrose. Observations were recorded weekly over a two month period.

Shoot production

Effect of growth regulators

In a preliminary experiment, the effects of different growth regulators on shoot production were assessed. Explants with mature somatic embryos were transferred to tubes containing ½ MS medium supplemented with either 0.5 mg l^{-1} thidiazuron (TDZ), 0.25 mg l^{-1} indole-3-butryric acid (IBA), 1 mg l^{-1} each of gibberellic acid (GA_3) and 6-benzyladenine (BA), or 1 mg l^{-1} kinetin (K) and 2.5 mg l^{-1} of 1:4 silver nitrate: silver thiosulphate (STS). For each treatment there were nine cotyledons and three replicates.

Effect of gibberellic acid treatment

The effect of GA_3 concentration on shoot production was examined. Explants with somatic embryos were cultured on MS containing GA_3 for 4-8 weeks. In the first set of experiments, explants with torpedo and cotyledonary-stage somatic embryos were cultured on 0.01, 0.1, 0.3, 0.5, 1, 3, 5, and 10 mg l^{-1} GA_3. Explants were cultured in tubes and there were 19-28 tubes per treatment. After two and a half weeks, the number of explants with shoots was recorded. Data were plotted as the percentage of explants with shoots for each treatment. In a second set of

experiments, explants with mature somatic embryos were cultured in deep plates containing 1, 3, 5, and 10 mg l^{-1} GA_3. There were approximately six explants per plate with 4-5 replicates per treatment. Observations on somatic embryogenesis were made after four and a half weeks and the data were plotted as the average percentage of explants with embryos and the average number of shoots per explant. Variability is represented as the standard deviation between replicates in each treatment after arcSIN transformation.

Shoot elongation

Shoots were elongated on ½ strength MS medium. The effects of various concentrations of BA (0, 0.5, 1.0, 2.0, and 5.0 mg l^{-1}) and GA_3 (0, 0.1, 0.5, and 1.0 mg l^{-1}) in combination were examined. For each treatment combination, there was a callus-derived explant with germinating somatic embryo(s) and/or tiny shoot(s) (~2 mm). The explants were cultured in glass tubes and there were 8 replicates per treatment combination. The degree of shoot elongation and general appearance of the shoots were recorded after one month. Four BA/GA_3 treatment combinations were also examined (0.1/0.5, 0.1/2.0, 0.1/1.0, 0.5/2.0 mg l^{-1}). The explants in this experiment were cotyledons from zygotic embryos that had germinating somatic embryos and small shoots (~2 mm).

Tissue recycling

Petiole and leaf explants, from *in vitro*-grown plantlets, were cut into 5-mm long and 10 mm^2 pieces, respectively and cultured on MS media containing various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 0.5, 1.0, 3.0 and 6.0 mg l^{-1}) and kinetin (0, 0.1, 0.5, and 1.0 mg l^{-1}). Callus formation and somatic embryogenesis were assessed after 7 weeks of culture. There were five explants of each tissue type per plate for each treatment combination and the experiment contained two replicates. A third replicate, with 4 petiole, 4 leaf, and 1 root explant per treatment combination was included but this replicate was kept in the dark for eight weeks. Explants with embryogenic calli and/or somatic embryos were transferred to growth regulator-free MS medium after 8-10 weeks for further embryo production and maturation. The effect of genotype was also evaluated in subsequent experiments, where petiole and leaf explants were cultured on MS medium containing 1.5 mg l^{-1} 2,4-D.

Petiole and leaf explants from *in vitro*-grown seedlings were cultured on MS medium containing 1.5 mg l^{-1} 2,4-D for ten weeks. Explants with large amounts of friable callus were transferred to 125 ml flasks containing 40 ml of liquid MS and a range of 2,4-D concentrations (0, 0.01, 0.1, 0.5, and 1.0 mg l^{-1}) and shaken at 125 rpm to produce cell suspension cultures. There were approximately 10 explants per flask (explants were weighed and distributed across flasks so that

weight per flask was consistent). Cultures were maintained by adding 10 ml of old suspension to 30 ml of fresh medium. After four weeks cultures were examined for somatic embryos.

Root production, acclimatization, and field studies

Elongated shoots were rooted on ½ strength MS medium in 25 mm diameter tubes or 6 cm x 6 cm x10 cm polycarbonate Magenta boxes. The effects of 1-naphthalenacetic acid (NAA) on rooting were assessed in two different experiments. These covered a range of concentrations: 0, 0.1, 0.5, 1, 3, 5, and 10 mg l^{-1}. Once shoots had formed well developed roots, they were potted in small fiber pots containing moistened Promix, covered with a plastic bag, and placed in a growth chamber held at 10°C under low light conditions. After two weeks, the bags were removed briefly on a daily basis, over a period of 2-3 weeks. After 4-5 weeks the bags were removed and the plants were allowed to senesce. Once senesced, the shoot portion of the plants was removed and if the root had formed a dormant bud it was either artificially wintered (stored in slightly moistened Promix at 4°C for 4 months) or planted in the field. Field performance was examined by comparing spring emergence and morphology of *in vitro*-derived plants with seed-derived plants. *In vitro*-derived roots with buds were planted in a low density plot and seeds were planted in a low and high density plot in the fall of 1998. A second field trial was planted in the fall of 1999. In this experiment, a plot of roots with buds from one-year-old senesced seedlings was included.

RESULTS AND DISCUSSION

A laboratory-scale micropropagation system based on somatic embryogenesis and multiple shoot formation has been developed for North American ginseng. This is the first complete summary on the performance of this species from introduction into culture to acclimatization and introduction into the field (see Figure 1).

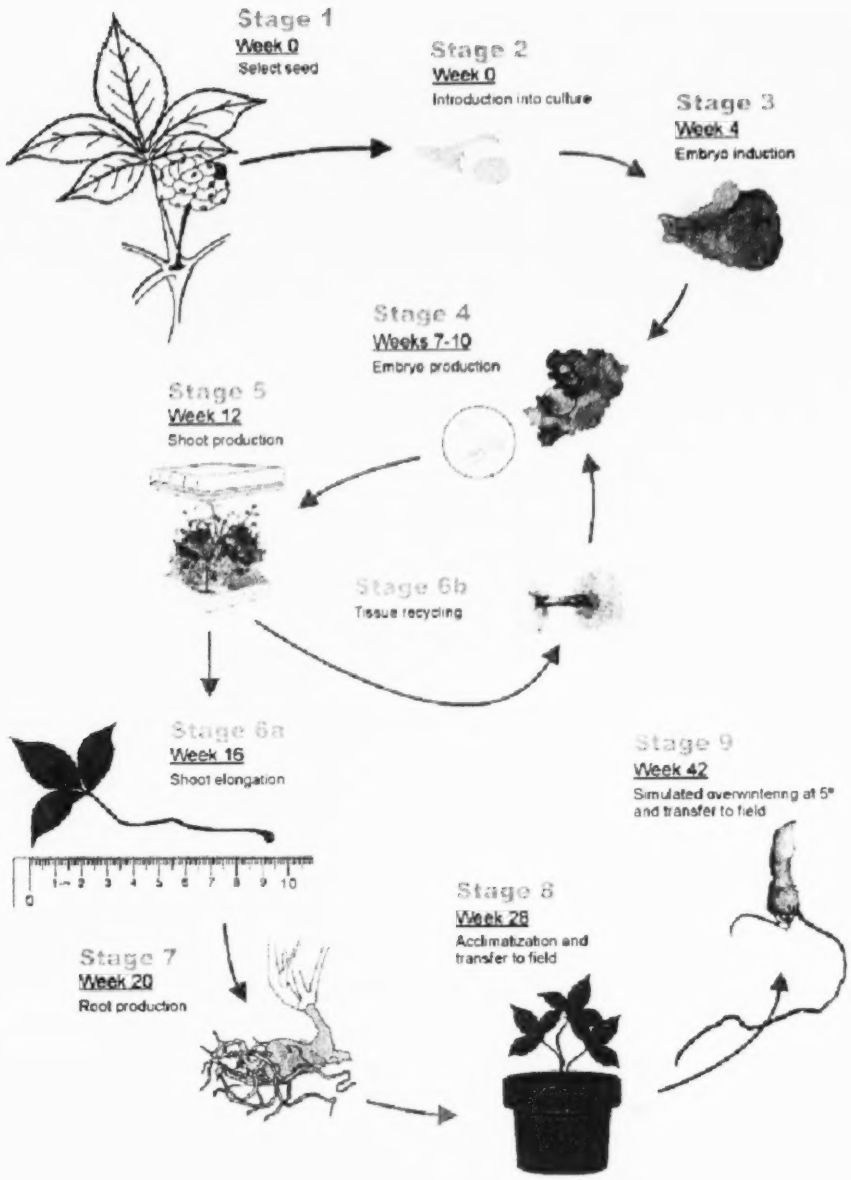


Figure 1. Nine-stage micropropagation protocol for *Panax quinquefolius*

Stages 1- 2: Germplasm selection and introduction into culture

Molecular studies have indicated that there is a high degree of genetic diversity within the North American ginseng population (Bai et al., 1997). Therefore, to create a stock of superior germplasm, plants must first be evaluated for characteristics such as ginsenoside content, disease resistance, root shape, and plant architecture. Plants with superior characteristics can then be multiplied rapidly by micropropagation.

Genetic diversity of field grown ginseng is also evident from the high variability seen in the experiments presented here. For instance, it was observed that cotyledons from the same zygotic embryo would respond in a similar fashion, in that they would swell to the same degree and tended to have the same degree of greening and/or anthocyanin production. Furthermore, while somatic embryos were induced on most cotyledon explants (Fig. 2a), the time of induction as well as the number of embryos produced per explant was variable (Fig. 2b). In an experiment where 46 cotyledon pairs were put on different treatments and tracked for six weeks, close to 70% of the pairs responded in the same way in that they either both produced somatic embryos or both had no somatic embryos, again indicating a genotype effect.

Because North American ginseng is plagued by a number of fungal diseases, it was necessary to soak seeds in a fungicide prior to sterilization with ethanol and bleach. Most of the seeds used in the experiments had red streaking indicating the presence of fungal hypae. While bacterial and fungal contamination of plant tissue can be a problem due to the outgrowth of endemic microorganisms, in the majority of the experiments it was only 1%. In some instances, contamination rates were as high as 20% but this was usually attributed to a "poor" batch of seed ("poor" seed was considered to be seed that had significant visible damage and showed visible growth of fungal and /or bacterial colonies.

Stages 3- 4: Somatic embryo induction and production

Cotyledons were approximately 5-6 mm at the start of culture. Excess sucrose in the culture medium stressed the explants and they expanded to nearly twice their size in the first two weeks. Somatic embryos began to form on the lateral edges and adaxial surface of the explants after four weeks. By the seventh week up to 90% of the explants had somatic embryos (Fig. 2a), with an average of 15-22 embryos per explant (Fig. 2b). Although, somatic embryos were induced on most of the explants, high variability in the number of somatic embryos per explant indicated that response efficiency is dependent on genotype. Culturing the explants in the dark decreased the percentage with somatic embryos at all sucrose concentrations (results not shown).

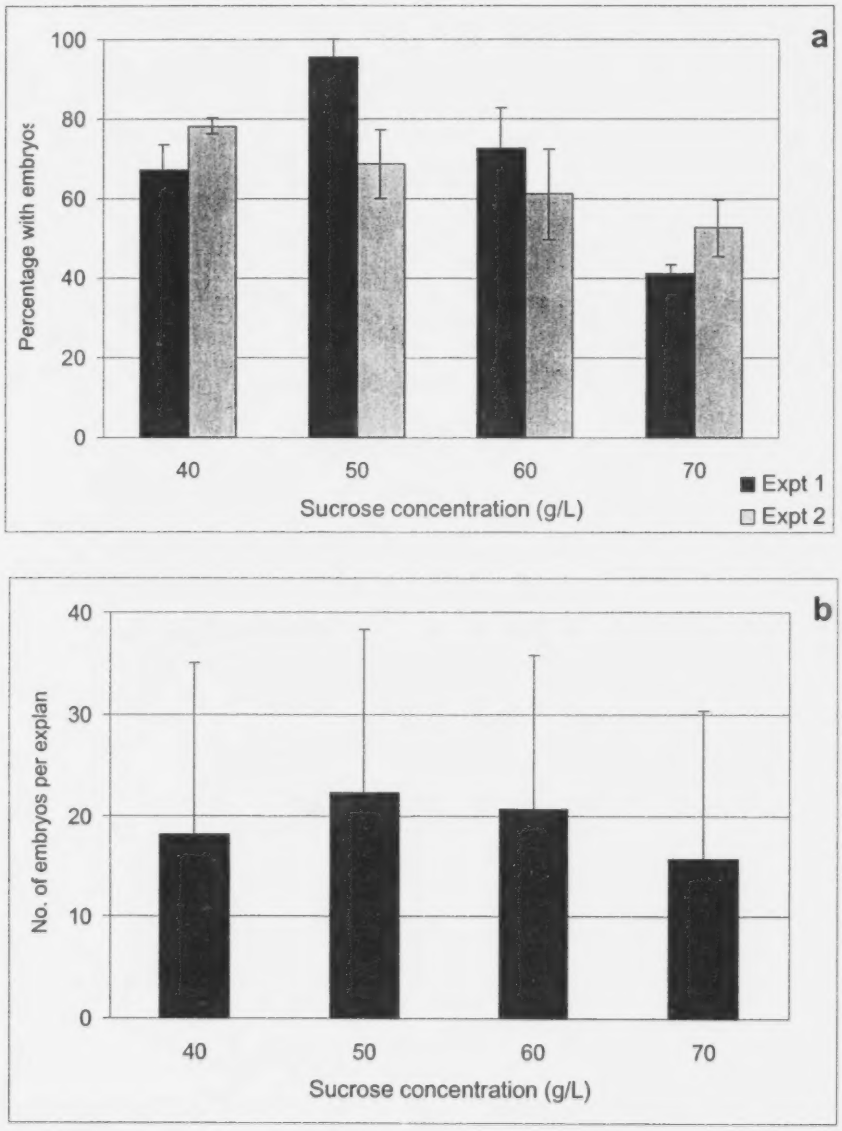


Figure 2. Effects of sucrose concentration on somatic embryo
(a) induction and
(b) production after seven weeks of culture.

While somatic embryo production was relatively high, with some explants producing as many as 90 embryos, most of the resulting embryos were structurally and/or developmentally abnormal (Figure 3 e-g). Abnormalities included precocious germination, secondary embryogenesis, de-differentiation, embryo fusion, extra cotyledons, and fused cotyledons. Such abnormalities are not uncommon in somatic embryo culture because seed tissues, which are important for normal embryo development, are absent and the *in vitro* environment is also much different from that of a developing zygotic embryo (Gray and Purohit, 1991). One of the most common observations is asynchronous development of somatic embryos from proembryonal masses (Gray and Purohit, 1991). Since somatic embryos are initiated at different times, even the smallest of changes in the culture environment can lead to drastic differences in embryo maturation. Therefore, a single explant can have somatic embryos at all stages of development.

Exogenous application of abscisic acid (ABA) has been used to promote normal development of somatic embryos in several species. In particular, ABA has been used to increase the frequency of embryos reaching maturity, prevent secondary embryogenesis, and eliminate structurally abnormal embryos such as those with fused or multiple cotyledons. Molecular studies have indicated that exogenous application of ABA can induce late-stage embryo genes (Busk and Pages, 1998).

Because of the importance of ABA in normal embryo development, it was of interest to see whether ABA could alleviate some of the abnormalities associated with somatic embryogenesis in *P. quinquefolius*, particularly with respect to asynchronous development and secondary embryogenesis. The addition of exogenous ABA did not affect embryo induction at 0.25 and 2.5 mg l^{-1}; however, at the highest concentration tested, 25 mg l^{-1}, it had an inhibitory effect (Fig.4a). The average number of embryos per explant decreased as the concentration of ABA increased but there was considerable variability at all concentrations, indicating a genotype effect. Unfortunately, at the concentrations tested, ABA was unable to synchronize development; the most highly embryogenic explants for each treatment all had somatic embryos at different developmental stages. There was evidence though, that 2.5 mg l^{-1} ABA may suppress secondary embryogenesis. At this concentration, just over half of the explants with more than 10 embryos had secondary embryos, compared to over 80% for the control and 0.25 mg l^{-1} treatment (Fig. 4b). ABA did not suppress abnormalities such as multiple cotyledons and embryo fusion.

Choi et al. (1998a), found that somatic embryos were abnormal when induced from cotyledons of *P. ginseng* cultured on MS medium with 5% sucrose. These abnormal embryos were referred to as "multiple" embryos and could not be regenerated into normal plantlets, either because they

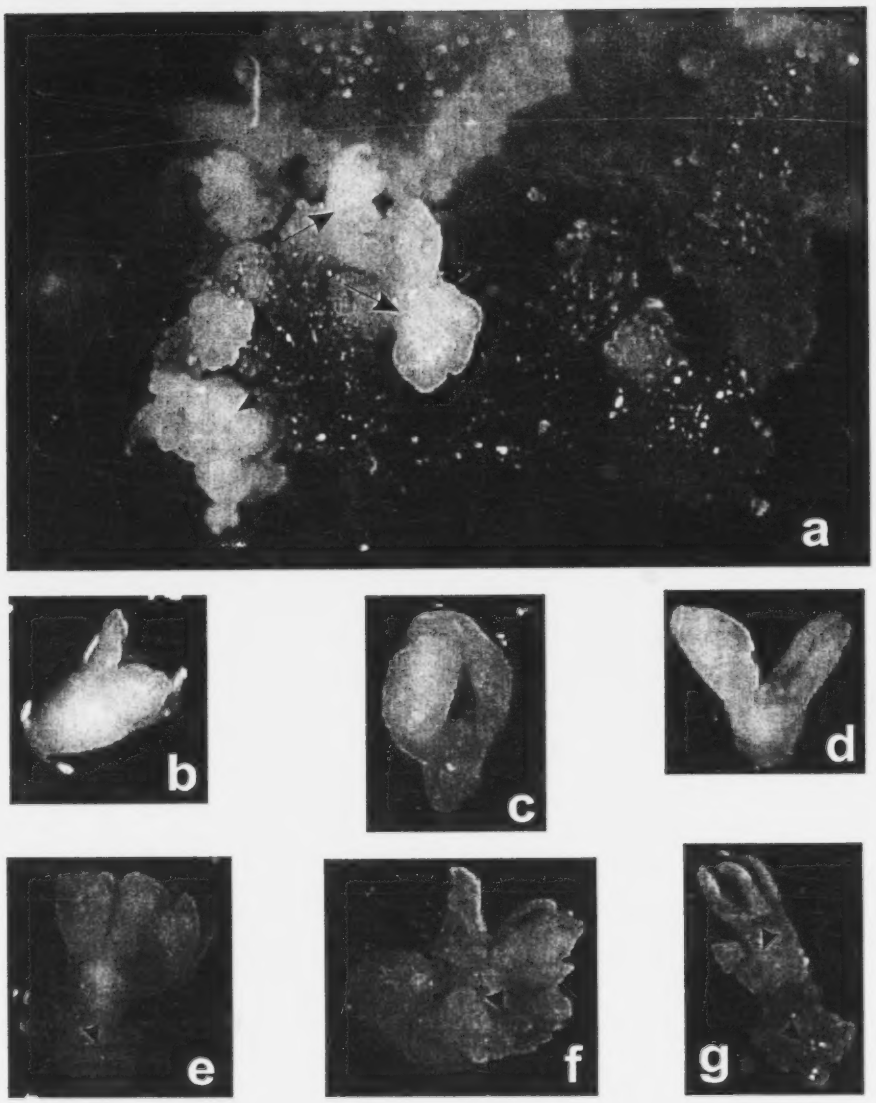


Figure 3. Types of somatic embryos **(a)** friable callus with somatic embryos (arrows), **(b)** late torpedo-stage embryo, **(c)** and **(d)** mature cotyledonary-stage embryos and **(e), (f),** and **(g)** abnormal somatic embryos (secondary embryogenesis Indicated by arrowheads).

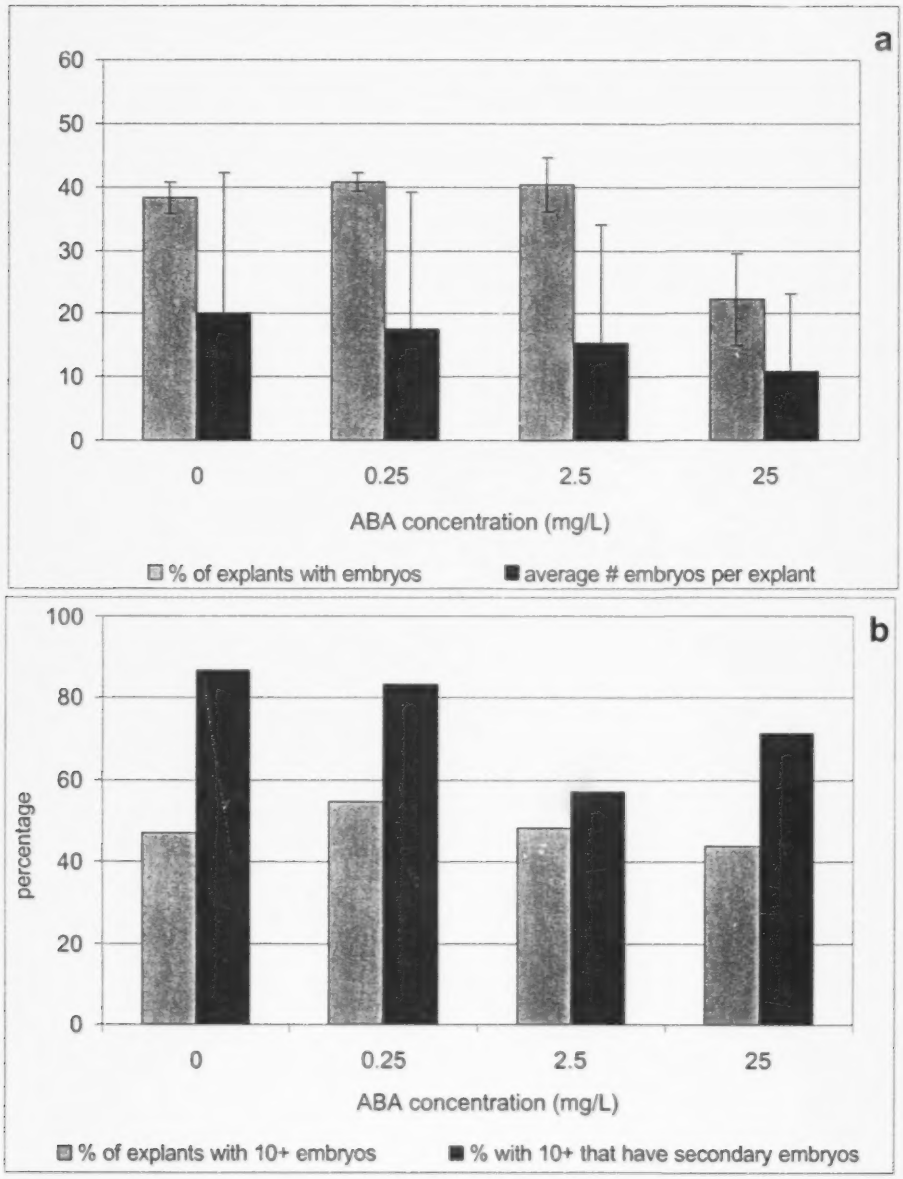


Figure 4. Effects of ABA concentration on **(a)** somatic embryo induction and production and **(b)** secondary embryogenesis from cotyledon explants after 3 weeks of culture.
Note: cotyledons were pre-cultured on control medium (0 mg L^{-1} ABA) for 3 weeks.

lacked a root meristem or the root meristem was fused to the parent explant. Choi et al. (1999) increased the frequency of normal somatic embryos by pre-treating cotyledon explants in a high sucrose solution (1.0 M) for 24-72 hours. Somatic embryos induced from the optimal pretreatment time of 72 hours, were all "single" (originating from a single somatic cell) and had a shoot and root meristem. Furthermore, the plasmolysis pretreatment resulted in a four-fold increase in the number of somatic embryos produced. The rational for this work was that the high sucrose pretreatment would physiologically isolate the cells of the explants by disrupting the intercellular communication channels (i.e, plasmodesmata) allowing somatic embryos to develop from single cells.

When cotyledon explants from *P. quinquefolius* were pretreated in a 1.0 M sucrose solution, the explants remained white, did not increase in size, and did not produce any somatic embryos after months of culture, while over 30% of the untreated cotyledons had somatic embryos after seven weeks. It is possible that the lack of response was the result of pre-treating the explants on semi-solid medium. However, in a second plasmolysis experiment where petiole and leaf explants were pretreated in an aqueous 1.0 M sucrose solution, the only sign of response was bleaching of some of the plant tissue. The effect of pre-treating cotyledon explants in an aqueous solution must be assessed in future work; however, it appears that *P. quinquefolius* differs from *P. ginseng* with respect to the effectiveness of this treatment..

Stages 5- 6a: Shoot production and elongation

Wang (1990) examined the effects of five different growth regulators (BA, GA_3, IBA, K, and NAA) on shoot production from somatic embryos of *P. quinquefolius*. While GA_3 gave the highest response (89%), plantlets originating on medium containing GA_3, BA, or K were hyperhydritic ("watery") and abnormal. On the other hand, normal, but slow-growing shoots were obtained when the explants were cultured on medium containing IBA or NAA. Based on these results, Wang modified the regeneration medium so that it contained a combination of IBA and NAA. When calli with somatic embryos were cultured on the modified medium, healthy shoots were obtained from 30% of the explants. In a preliminary experiment, reported here, four different growth regulator-supplemented media were tested for their ability to promote shoot maturation of somatic embryos. Medium containing a combination of GA_3 and BA gave the optimal response with 96% of the explants producing shoots. This medium also gave the most normal looking shoots. Contrary to Wang's results, IBA not only gave the poorest response (3.5%) but none of the resulting shoots showed normal morphology.

Somatic embryos induced from all explant sources tested, i.e., cotyledons, petioles and stems, leaves, and roots, were dormant or quiescent and would not germinate unless transferred to media containing gibberellic acid. These results are consistent with studies on *P. ginseng*. Choi et al. (1999) report that somatic embryos induced from cotyledon explants of *P. ginseng* require a GA_3 treatment (or eight weeks of vernalization) in order to germinate. In a range of GA_3 concentrations (0, 1 , 4, 8, and 10 mg l^{-1}) and found that while germination will occur on medium containing 1 mg l^{-1} GA_3 while higher concentrations are necessary for close to 100% germination. Choi et al. (1998a) and Choi et al. (1998b) used 10 mg l^{-1} GA_3 to germinate somatic embryos of *P. ginseng* while Kishira et al. (1992) have reported that lower concentrations (e.g., 0.5 mg l^{-1}) result in better quality shoots. Other studies reported promotion of shoot production from somatic embryos of *P. ginseng* with a combination of GA_3 and BA (Arya et al., 1991; Lee et al., 1990).

When somatic embryos of *P. quinquefolius* were cultured on full-strength MS in the presence of as little as 0.1 mg l^{-1} GA_3 shoots were produced within four weeks. Response to the GA_3-containing medium was obvious in the first week of culture by the greening and elongation of the cotyledons and germination of some of the somatic embryos within the first few days. Shoot production was highly efficient with explants cultured on medium containing 1 mg l^{-1} or greater GA_3, occurring on up to 100% of the explants (Fig. 5a) with an average of 18-20 shoots per explant (Figure 5b). As the shoots proliferated, they were broken apart and cultured on ½ MS containing a combination of BA and GA_3. Once elongated, the shoots were either rooted or recycled. In addition, use of increased GA_3 levels also promoted embryo maturity and root development. As shown in Figure 6, the number of cotyledons with elongated shoots and roots increased concomitantly with the increase of GA_3 in the nutrient medium.

Stage 6b: Tissue recycling

In vitro shoots and plantlets were recycled by inducing embryogenic callus from stem and petiole (Fig. 3a), leaf, and root cuttings and then reintroducing the explants at Stage 3 of the protocol (see Fig. 1). Somatic embryos could not be induced directly from these tissue types by introduction at Stage 1 of the protocol. When cultured in the presence of excess sucrose, these explants remained unresponsive eventually turning brown and dying. Studies on callus cultures of the *Panax* species report the induction of embryogenic callus, from a variety of explant sources cultured on medium containing 2,4-D or a combination of 2,4-D and K (Shoyama et al, 1997; Shoyama et al. 1995; Arya et al., 1991; and Lee et al., 1990). In most of the studies on *P. ginseng* (see Appendix 1), callus induction was optimal when 2,4-D concentration was 1 mg l^{-1}. Choi et al (1997) conducted experiments that tested the effects of different auxins and cytokinins, alone and in combination, on callus production in a similar species, *Acanthopanax koreanum* Nakai.

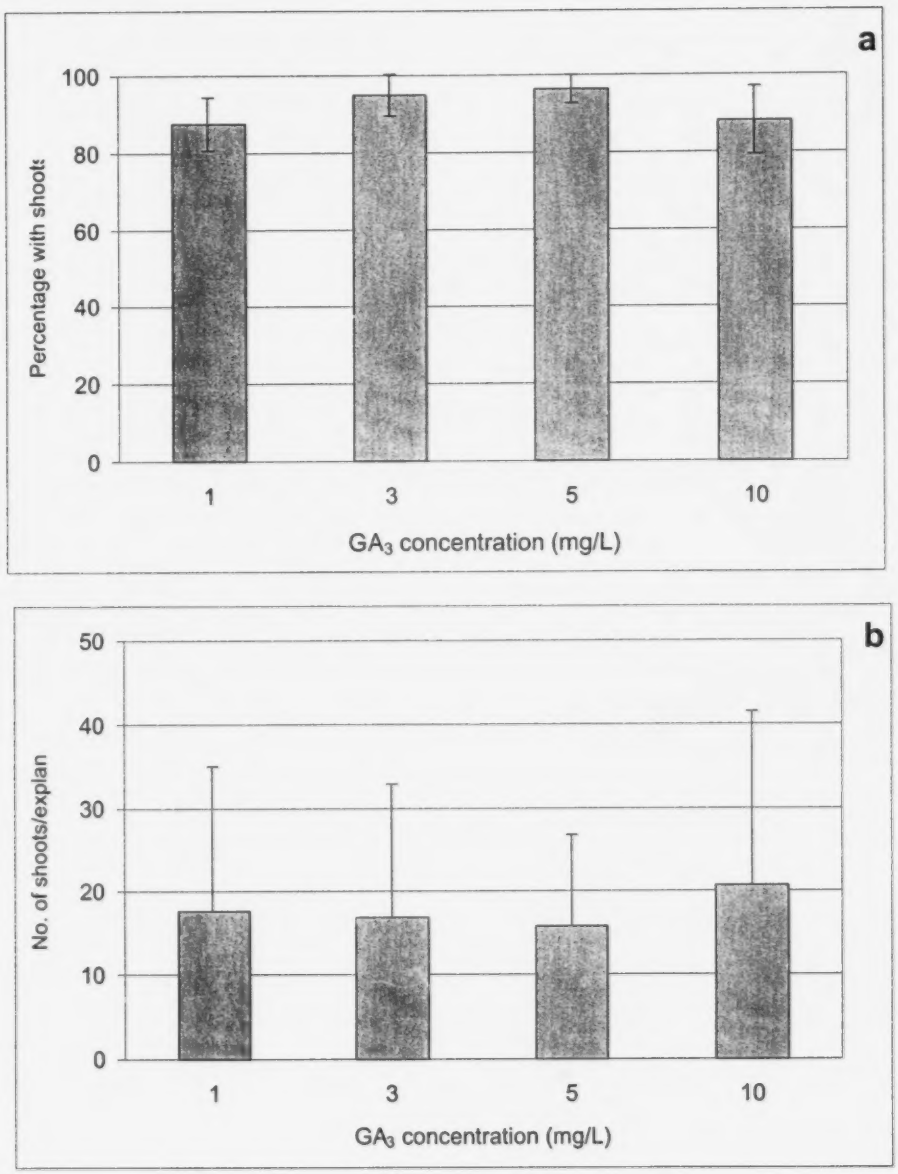


Figure 5. Effects of GA_3 concentration on (a) shoot regeneration and (b) production from cotyledon explants with somatic embryos after four weeks of culture.

This demonstrated that auxin, in the form of 2,4-D appears to be necessary for inducing embryogenic callus, and that the optimal concentration is also 1 mg l^{-1} 2,4-D. Higher 2,4-D concentrations (i.e., 5 mg l^{-1}) have been shown to be optimal for embryogenic callus production from explants of *P. notoginseng* (Kishira et al., 1992). In *P. quinquefolius*, Wang (1990) reported that embryogenic calli could be induced from root explants within four weeks when explants were cultured on medium containing 2 mg l^{-1} 2,4-D and 1 mg l^{-1} K.

Preliminary experiments, followed by later studies (see Fig. 7) conducted as part of this study, testing a range of 2,4-D and K concentrations, indicated that the optimal 2,4-D concentration for inducing somatic embryos was between 1 and 3 mg l^{-1}. This is consistent with Wang's (1990) findings for *P. quinquefolius*; however, our results did not show K to be an important factor and is in contrast to other studies with *P. ginseng*. Choi et al. (1997) report that while cytokinins increased the fresh weight of callus from *A. koreanum*, they did not promote embryogenic callus or somatic embryo formation. Based on our results and on information from the literature, we have chosen 1.5 mg l^{-1} 2,4-D for inducing embryogenic callus from explants of *in vitro* plantlets and shoots of *P. quinquefolius* for recycling (see Figure 1, Stage 6b). Using this concentration, callus was induced on up to 100% of petiole explants in as early as four weeks.

Choi et al. (1997) found also that somatic embryos induced from callus developed asynchronously. Therefore, to obtain embryos at the same developmental stage they cultured embryogenic callus in liquid and then used a sieve to separate globular embryos from more mature embryos. We have tested their protocol on embryogenic callus from *P. quinquefolius* but currently it is not efficient and the globular embryos that are obtained from liquid culture do not mature and develop into shoots; thus, this approach was not persued.

The source of explant proved to be important for recycling efficiency. Of the explants tested, petioles showed the highest response; petioles from about 47% of the shoots formed somatic embryos (Table 2). It should be noted, however, that the percentage of explants that actually produced somatic embryos was much lower (15.6%) and that embryogenic efficiency was genotype dependent. Somatic embryos were also induced on callus from leaf and root segments but at much lower frequencies. In addition, the results shown in Table 2 show that there was a strong correlation between the initial *in vitro* embryo production response (Stage 4) and subsequent responses during recycling (Stage 6b). This suggests there may be a strong genetic basis to the *in vitro* embryo formation response.

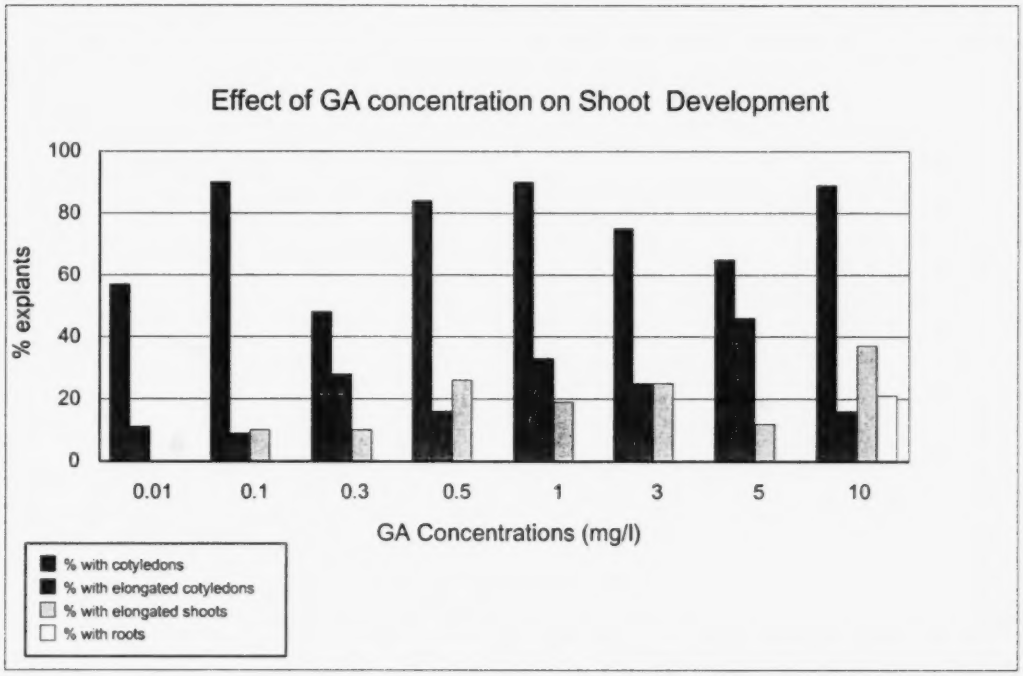
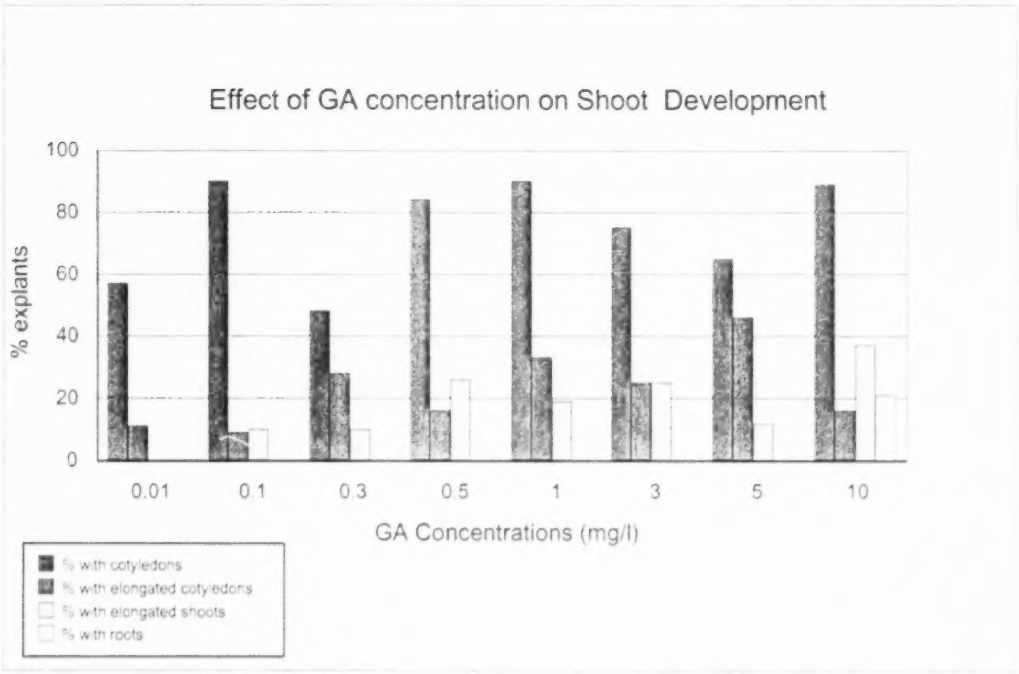


Figure 6. Effect of GA3 on somatic embryo shoot development and root induction.

Table 2. Callus production and somatic embryogenesis from petiole explants of *in vitro* shoots.

*Response category	Total # of shoots	Total # of explants	CALLUS: Total # with	CALLUS: %with	SOMATIC EMBRYOS: Total # with	SOMATIC EMBRYOS: % with	% of shoots that responded
None	71	383	284	74.15	0	0	
Low (1-20)	19	162	131	80.86	22	13.58	
Med (21-50)	19	107	98	91.59	42	39.25	
High (51-100)	13	66	64	96.97	48	72.73	46.66
Totals	122	718	577	80.36	112	15.6	

*Response category refers to whether the explants came from a shoot that showed a high, medium, or low embryogenic response with respect to the percentage of explants from that shoot that produced somatic embryos.



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Stage 7: Root production

There is little information in the literature on *in vitro* root development of *Panax* species. In most reports, elongated shoots are rooted on MS medium with reduced salts, with little detail given on the efficiency or quality of the roots. Choi et al. (1998b) investigated the effect of nitrogen on root production in *P. ginseng* by culturing elongated shoots on MS medium with various concentrations and combinations of ammonium nitrate (NH_4NO_3) and potassium nitrate. (KNO_3). They found that root growth was enhanced when NH_4NO_3 was omitted from the medium and when KNO_3 potassium nitrate was present in reduced quantities. However, when elongated shoots of *P. quinquefolius* were cultured on modified-MS medium there was no apparent effect on rooting.

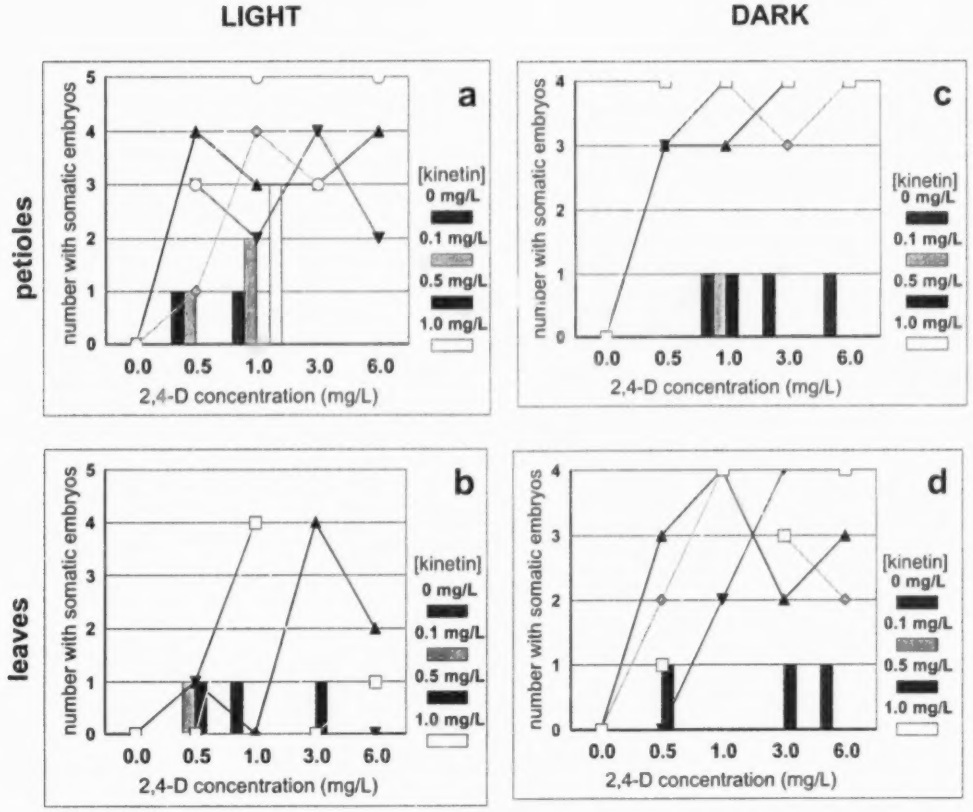

Figure 7. The effect of 2,4-D and kinetin on callus production (lines) and somatic embryo formation (bars) after seven weeks of culture from **(a)** petioles and **(b)** leaves and after eight weeks of culture in the dark for **(c)** petioles and **(d)** leaves. **Note**: For each treatment combination, n=5 for (a) and (b), and n=4 for (c) and (d).

Auxins are also known to promote rooting in several plant species. Shoyama et al. (1997) reported that NAA was optimal for rooting in *P. ginseng* compared to other auxins (IBA and IAA). The effects of three auxins (2,4-D, IBA, and NAA) on *P. quinquefolius* were examined showing that NAA was the best auxin for inducing roots (results not shown). However, in all experiments, rooting was only possible when there was a root meristem present. Rooting was observed on ½ MS medium, in the absence of plant growth regulators (Fig. 8) but when NAA was added to the medium at low concentrations, the percentage of explants with roots increased, as did the number of roots per explant. At higher NAA concentrations (i.e, >3 mg l^{-1}), almost all of the explants formed roots but these were accompanied by callus formation, hyperhydration and browning towards the basal ends of the shoots.

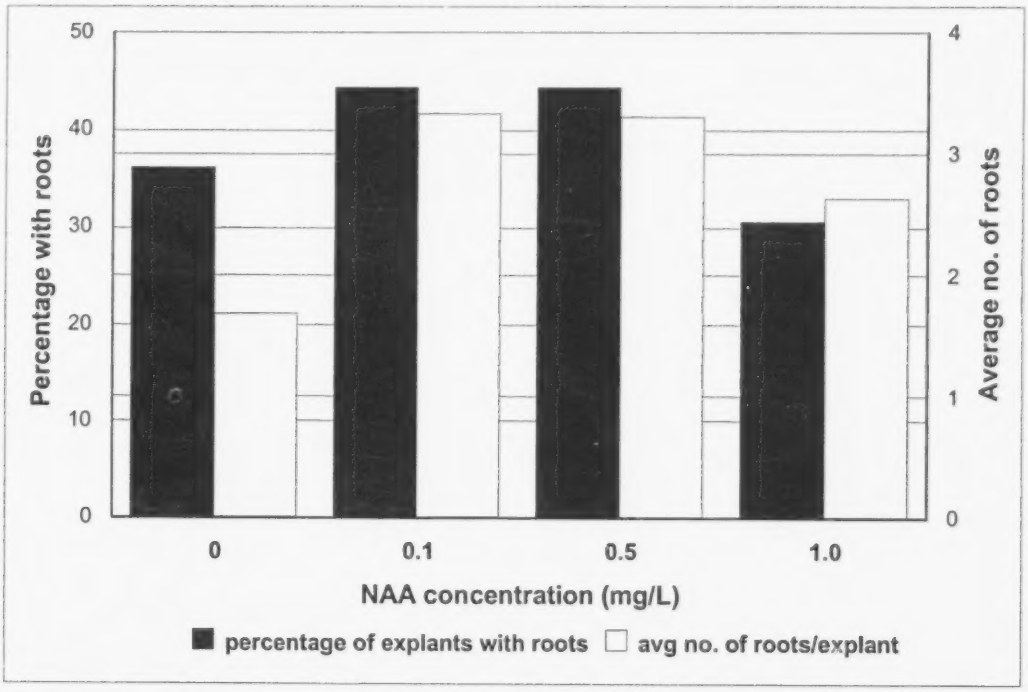

Figure 8. The effect of NAA concentration on rooting after 2 months of culture.

Rooting appears to be genotype dependent in *P. quinquefolius* with respect to the ability to form roots as well as root quality. When shoots from the same explant were tracked, it was noted that some explants would form well-developed roots regardless of the treatment conditions. Furthermore, only a small percentage of shoots (~1%) produced good quality roots with taproot-like morphology. These observations are in line with those described in Table 2 which show a linkage between genotype and *in vitro* performance.

Stage 8: Acclimatization and transfer to field

Plantlets with well developed roots were acclimatized under low light conditions at 10°C for four to eight weeks. Of those plantlets acclimatized in 1999, 65% were considered to have acclimatized successfully having healthy roots with dormant buds at the time of senescence. As shown in Figure 10, the plantlets maintained a healthy status from stage 6 through 8 but did not appear to grow after being removed from culture.

Stage 9: Overwintering and transfer to field

Exposue of plants which entered dormancy after acclimation and transfer to soil (in the greenhouse) could be over wintered in growth chambers by covering with moist peatmoss and packing loosley in a plastic bag and left for 100 days at 5°C in the dark. Over 90% of dormant roots germinated after this treatment and (as shown in Fig. 9) resulted in larger and more vigourous plants than their stratefied seed controls. In addition these "first year" plants showed a high frequency of reduced flowering and seed set (see Fig. 9).

Results from the first overwintering field trial indicate that efficiency upon transfer needs to be improved. Only 11% of the tissue-culture derived plants emerged in the spring, compared to 62% for seed-derived material planted at the same density. The higher density plot with seed-derived material had a similar emergence rate of 63%. While the emergence rate was low for tissue culture-derived material, the tissue-derived plants resembled those of the seed-derived plants and roots appeared normal (see Fig. 11). It is possible that the late planting fall date affected field performance of the tissue-derived material.

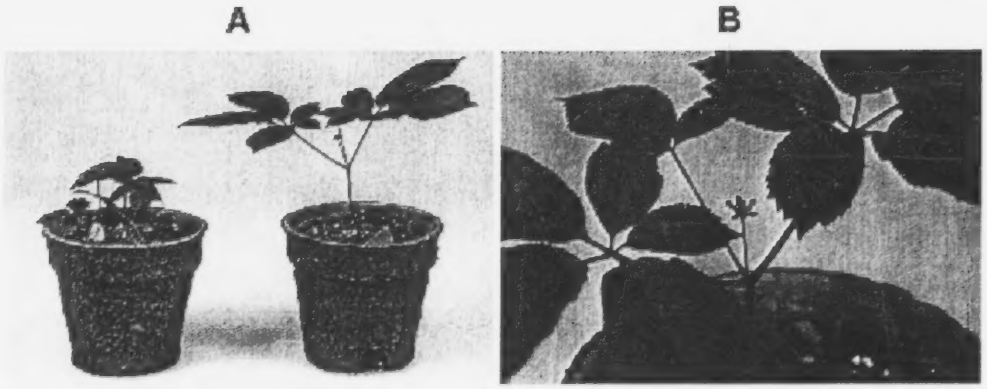

Figure 9. **A:** Comparison of 1st year stratefied seed-derived ginseng plants (left) and 1st year seedlings after the 100-day overwintering treatment (right). **B:** Reduced flower umbel on 100-day overwintered seedlings

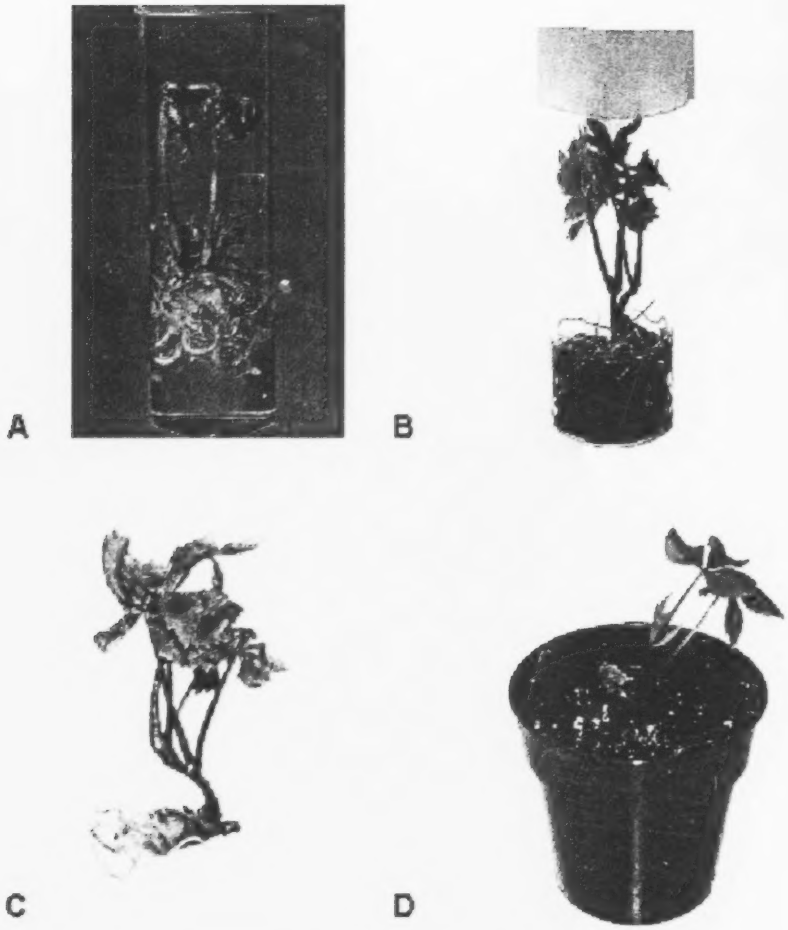

Figure 10: **A.** Multiple shoots at Stage 6a, **B**. Rooted shoots at Stage 7, **C.** Rooted shoot in B after removal from culture, **D.** Established plant after acclimation in the greenhouse.

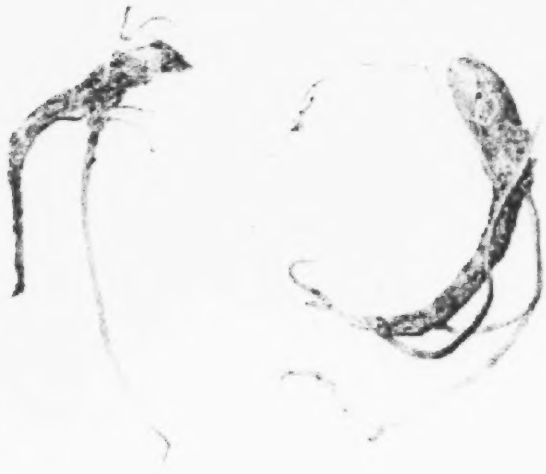

Figure 11. Field - overwintered tissue culture-derived plants (About 5 cm in length).

CONCLUSIONS

A laboratory-scale micropropagation system for *P. quinquefolius*, based on an 8-stage protocol, involving somatic embryogenesis and multiple shoot formation has been developed. The optimized protocol and is summarized below:

Stage 1 (Week 0): Plant and seed selection. The response in tissue culture is strongly genotype dependent; that is, each plant responds differently. Tracking of individual plants show that each cotyledon pair (explant) respond in the same way and a group of recovered plants can be traced back to a single responding plant in a particular batch of seed.

Stage 2 (Week 0): Introduction into culture. Bacterial and fungal contamination of plant tissue can be a problem due to the outgrowth of endemic microorganisms. Mid-mature seed-derived cotyledon tissue (explant) responds best and quickest. Petiole, stem, leaf, and root tissue explants will also form somatic embryos at lower frequencies, with petiole, tissue responding closest to that of the parental cotyledon explants.

Stage 3 (Week 4): Embryo induction. Cotyledon explants are approximately 5-6 mm at the start of culture. High sucrose in the culture medium stresses the explants and they expand to nearly twice their size in the first two weeks. Somatic embryos form on the lateral edges and adaxial surface of the explants after four weeks and, by the seventh week, up to 90% of the explants have somatic embryos with an average of 15-22 embryos per explant. There is a high variability in the number of somatic embryos per explant indicating that response efficiency is dependent on genotype.

Stage 4 (Week 7-10): Embryo development. One of the most common observations is asynchronous development of somatic embryos; since somatic embryos are initiated at different times, a single explant has somatic embryos at all stages of development.

Stage 5 (Week 12): Shoot production. Somatic embryos germinate within 4 weeks after transfer to medium containing the plant growth regulator gibberellic acid. Shoot production is highly efficient and occurs on up to 100% of the explants with an average of about 20 shoots per explant.

Stage 6a (Week 16): Shoot elongation. As the shoots proliferate, they are separated and transferred onto ½ strength Murashige and Skoog medium containing a cytokinin-type growth

regulator and gibberellic acid. Shoots elongate asynchronously but, conservatively; 10 shoots can be recovered from 90% of the explants.

Stage 6b (4 weeks): Tissue recycling. Leaf and petiole tissue from responding cultures can be cut into small pieces (explants) and reintroduced to culture. After 4 weeks on recycling medium, cell division is induced in the explants and the resulting callus explant is reintroduced into the protocol at Stage 4 for additional cycles of embryo induction. About 50% of the tissue can be successfully recycled in this manner to boost production.

Stage 7 (Week 20): Root production. Rooting appears to be genotype dependent and can occur on ½ strength Murashige and Skoog medium, in the absence of plant growth regulators. However, in all experiments, rooting was only possible when there was a root meristem present. Overall, root induction efficiency is about 50% but only 1% of plants have a good quality tap root-like morphology.

Stage 8 (Week 28): Acclimatization and transfer to field. Plants acclimatized under low light conditions at 10°C for four to eight weeks at a 65% efficiency rate. This rate can be improved.

Stage 9 (Week 42): Overwintering and transfer to field. Tissue culture-derived rooted plants perform best when, after establishment in soil plugs in the greenhouse, dormancy is induced and the young newly formed dormant buds are subjected to about 100 days of 5^0C temperature to mimic overwintering. Under greenhouse spring conditions, plants break dormancy and appear to produce normal-looking ginseng plants and roots.

FUTURE DIRECTIONS

Protocol efficiency for plant multiplication is high in the early stages of the protocol where more research studies have been accomplished to date. With a production efficiency of 19 shoots per explant on 90% of the explants after 16 weeks, there is the potential for dramatic scale-up numbers. Shoot elongation, rooting efficiency, and survival in the field must be improved. At present, quality root formation on the tissue-culture derived-shoots is the limiting step. Ginseng plants after the simulated overwintering treatment flower in their first year of growth and resemble a two-year-old ginseng plant in size and leaf morphology. Present low field survival after transfer may be due to the late fall planting time that has been used in the preliminary tests (stratified control seed plantings also showed low survival rates); overwintering in a 100-day cold treatment followed by spring planting would likely dramatically increase field transfer rates.

Thus, future research activities should be focussed on:

1. Improvement of the latter stages of the *in vitro* propagation protocol.

2. Optimisation studies to develop an effective field transfer and overwintering protocol including mechanisation of the transplant technique.

3. Improvement of the efficiency of using mature explants from young field-grown plants.

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GLOSSARY

ABA:	Abscisic acid
BA:	6-Benzyladenine
2,4-D:	2,4-Dichlorophenoxycacetic acid
GA_3:	Gibberellic acid
IBA:	Indole-3-butyric acid
K:	Kinetin
L:	Liter
M:	Molar
mL:	Milliliter
MS:	Murashige and Skoog plant growth medium (Murashige and Skoog, 1962)
NAA:	1-Naphthaleneacetic acid
rpm:	Revolutions per minute
SCPFRC:	Southern Crop Protection and Food Research Centre
STS:	1:4 silver nitrate: silver thiosulphate
TDZ:	Thidiazuron

APPENDIX 1

Somatic embryogenesis in Panax and other ginseng-like species in the Araliacae family

Table 1.

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
***Panax ginseng* C. A. Meyer (Korean ginseng)**												
cotyledon	ZE	Embryogenic callus induction (4 wks):							nd	nd	unknown	Ahn 1996
		MS	none	sucrose (30)	none							
		SE induction from embryogenic cell clumps (4 wks):										
		MS	NH_4NO_3 : KNO_3 (mM):	sucrose (30)	2,4-D (.55)* + BA (.11)*	nd	8 wks total					
			0:10					0				
			5:10					7 ±1.8				
			5:20					9 ±1.2				
			5:40					12 ±2.8				
			10:10					3 ±1.2				
			20:20					2 ±0.5				
cotyledon transverse thin cell layers	*in vitro* seedlings:	MS	none	sucrose (30)	2,4-D (1.105)				plantlet-like structures remained dormant		nd	Ahn et al. 1996
	5 d					15 ±3	12 wks	2 ±1				
	10 d					22 ±2	9 wks	4 ±1				
	15 d					32 ±3	9 wks	8 ±2				
	15 $d^{2,4\text{-D pretrt}}$					40 ±3	7 wks	10 ±2				
	15 $d^{2,4\text{-D pretrt}}$				+ TDZ (.002)*	58 ±3	6 wks	8 ±3				
	15 $d^{2,4\text{-D pretrt}}$				+ BA + zeatin* (.02 each)	62 ±2	6 wks	8 ±2				
cotyledon	ZE (3mm)	MS	none	sucrose (30) Pretreatment sucrose (3000):	none	nd	2 mths		87	2 mths	unknown	Choi et al. 1999a
				for 72 hrs				37 ±5.7				
				for 48 hrs				36 ±6.4				
				for 24 hrs				23 ±5.6				
				0 hrs (control)				9 ±2.7				
cotyledon[a] [b]	ZE:	MS	none	sucrose (50)	none		2 mths	nd	nd	nd	unknown	Choi et al. 1998b
	(6mm)					66 ±5						
	(3mm)					94 ±7						
	(1mm)					0						

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
cotyledon[a]	ZE (6mm)	MS	none	sucrose (50)	none	1266	5 days-1mth	nd	n/a	n/a	n/a	Choi et al. 1997a
cotyledon[b]												
cotyledon[a]	ZE (6mm)	MS	none	sucrose (30)	none	68	3 wks	nd	n/a	n/a	n/a	Choi et al. 1997b
[d]												
***Panax ginseng* C. A. Meyer (Korean ginseng)**												
cotyledon	ZE (4.2mm)	MS	none	sucrose (30)	none	76.4 ±5.4	nd	nd	n/a	n/a	n/a	Choi & Soh 1996
cotyledon	ZE:	MS	none	sucrose (30)	none		5 wks	nd	n/a	n/a	n/a	Choi & Soh 1995a
	(6mm)					0						
	(4mm)					0						
	(3mm)					12.1 ±2.4						
	(2mm)					76.4 ±5.4						
	(1mm)					0						
cotyledon	ZE (4mm)	MS	none	sucrose (30)	none (control)	~74	2-3 wks	nd	n/a	n/a	n/a	Choi & Soh 1995b
					IAA (1)	~42						
					2,4-D (1)	~35						
					K (1)	~2						
					BA (1)	~10						
cotyledon[a]	ZE:	MS	none	sucrose (30)	none		4 wks		n/a	n/a	n/a	Choi & Soh 1994
[b]	(6mm)					~38		11.3 ±7.24				
	(4mm)					~78		16.6 ±9.32				
	(3mm)					~87		9.7 ±3.66				
	(2mm)					92.7 ±10.3		7.5 ±3.25				
***Panax quinquefolius* L. (North American ginseng)**												

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
cotyledon	ZE	MS	none	sucrose:	none		6 wks					present study
				(0)		0		n/a				
				(20)		0		n/a				
				(40)		13.33 ±2.79		0.87 ±0.82				
				(50)		46.67 ±2.34		6.90 ±4.58				
				(60)		36.67 ±2.58		9.13 ±5.72				
				(70)		70.00 ±3.87		17.23±2.6				
				(80)		66.67 ±0.88		11.03±1.1				
				(100)		76.67 ±0.74		12.37±4.1				
***Panax ginseng* C. A. Meyer (Korean ginseng)**												
cotyledon	ZE	MS	none	sucrose (30)	none					n/a	n/a	present study
				Pretreatment sucrose (3000):								
				72 hrs			6 wks					
				24 hrs		2.25 ±0.58		0.12 ±0.77				
				none (control)		21.67 ±1.89		2.05 ±0.58				
						21.67 ±1.13		2.26 ±7.77				
ZE	n/a	Callus and primary SE induction (4-6 wks):							80	~7-12 wks	unknown	Arya et al. 1993
		MS	none	sucrose (30)	2,4-D (1) and K (.01)	nd for PSE	6 wks	nd		(20-25 wks from start of culture)		
		Induction of adventitious SE from primary SE (5-7 wks):										
		MS	none	sucrose (30)	2,4-D (1)*	8060205	11-13	8 ±2.1				
					NAA (1)		wks	6 ±1.6				
					IAA (1)		total	5 ±1.0				
					K/BA (1 each)			2 ±0.6				
ZE	n/a	Callus and SE induction (6 wks):										
		MS	none	sucrose (30)	2,4-D(1) + K(.1)	nd	6 wks	nd	nd	nd	unknown	Arya et al. 1991

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
ZE (6mm)	n/a	MS	Optimal [salt]*: none (control) 3X NH_4NO_3 3-6X KNO_3 3X KH_2PO_3 3X $MgSO_4 7H_2O$ 3X $CaCl_2 2H_2O$ 2X total salts	sucrose (30)	none	 0 56.8 29.4 11.5 9.7 5.7 ~45	2 mths	nd	76	nd	unknown	Choi et al. 1998a
ZE (2mm) ZE (3mm) ZE(4'mm)	n/a	MS	none	sucrose (30)	none	76.412	5 wks	nd	n/a	n/a	n/a	Choi & Soh 1995a
***Panax ginseng* C. A. Meyer (Korean ginseng)**												
ZE (4mm)	n/a	MS	none	sucrose (30)	none 2,4-D (1) IAA (1) K (1) BA (1)	0 ~40 ~17 0 0	2-3 wks	nd	n/a	n/a	n/a	Choi & Soh 1995b
ZE	n/a	Induction of SE and callus:							nd	nd	unknown	Lee et al. 1990
		MS	none	sucrose (30)	none 2,4-D (1)* and K (.01)*	3055	6 wks	nd				
leaf transverse thin cell layers	*in vitro* seedling: (15 d)	MS	none	sucrose (30)	2,4-D (1.105)	12 ±2	9 wks	5 ±2	plantlet-like structures remained dormant		n/a	Ahn et al 1996
leaf lamina	3 y old plants	Embryogenic callus induction:										
		RM	none	sucrose (30)	NAA (2) and K (.5)	nd	6 mths	nd	n/a	n/a	n/a	Cellarova et al. 1992
leaf		Embryogenic callus induction:							nd	nd	n/a	Kishira et al. 1992

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
	in vitro shoot	MS	none	sucrose (30)	2,4-D (1)	4020	3 mths	nd				
	5 y plant											
leaf	*in vitro* plantlet	DIRECT ORGANOGENESIS							71.6% -normal shoots 40.5% -rooted		unknown	Lim et al. 1997
***Panax quinquefolius* L. (North American ginseng)**												
leaf		Embryogenic callus induction:							61	6 wks	unknown	Tirajoh et al. 1998
		MS	none	sucrose (30)	2,4-D (2) and NAA (1.8)*			nd				
	3-5 y old plant					303040	7 mths					
	in vitro seedling						2 mths 3 mths					
leaf	*in vitro* shoot	Embryogenic callus and SE induction:							nd	nd	nd	present study
		MS	none	sucrose (30)	2,4-D (1.5)-for the first 10 wks	6.2	15 wks	nd				
***Panax ginseng* C. A. Meyer (Korean ginseng)**												
petiole transverse thin cell layers	*in vitro* seedling: (15 d)	MS	none	sucrose (30)	2,4-D (1.105)	5 ±1	13 wks	3 ±2	plantlet-like structures remained dormant		n/a	Ahn et al 1996
stem		Embryogenic callus induction:							nd	nd	unknown	Kishira et al. 1992
		MS	none	sucrose (30)	2,4-D (1)		3 mths	nd				
	in vitro shoot					205						
	5 y old plant											

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
petiole	*in vitro* plantlet	DIRECT ORGANOGENESIS							71.6% -normal shoots 40.5% -rooted		unknown	Lim et al. 1997
***Acanthopanax koreanum* Nakai**												
stem	3 y old plant	Embryogenic callus induction (8 wks + 2 wks of liquid culture):							91% germ in 1-2 wks 85% elong. in 1 mth		unknown	Choi et al. 1997
		MS	none	sucrose (30)	2,4-D (1)*	12	10 wks					
		SE induction from suspended cell clumps (2 wks):										
		MS	none	sucrose (30)	2,4-D (.1)*	nd	12 wks	350/flask				
***Panax quinquefolius* L. (North American ginseng)**												
epicotyl	*in vitro* seedling	Embryogenic callus induction (3 mths):							0	n/a	n/a	Tirajoh et al. 1998
		MS	none	sucrose (30)	dicamba (2)* and K (1.08)*							
		SE induction from callus (6 mths):										
		MS	none	sucrose (30)	dicamba (2)*	2	9 mths	nd				
stem/pet.	*in vitro* shoot	MS	none	sucrose (30)	2,4-D (1.5)							present study
***Panax pseudoginseng* var. *notoginseg* (Sanchi ginseng)**												
petiole	field grown plants	Embryogenic callus induction:							85% formed shoots		n/a	Shoyama et al. 1997
		MS	none	sucrose (30)	2,4-D (1)	nd	5'mths	nd				
***Eleutherococcus senticosus* Maxim. (Siberian ginseng)**												
ZE	n/a	MS	none	sucrose (30)	2,4-D (1)	nd	nd	nd	nd	nd	n/a	Choi & Soh 1993 (Genus is referred to as *Acanthopanax*)
ZE	n/a	MS	none	sucrose (30)	2,4-D (.5)	83.3	1-2mts	15-21	75	r-d	unknown	Gui et al. 1991 (Genus is referred to as *Acanthopanax*)
***Panax ginseng* C. A. Meyer (Korean ginseng)**												

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
flower bud		Embryogenic callus induction:							nd	1 mth	unknown	Kishira et al. 1992
		MS	none	sucrose (30)			3 mths	nd				
	in vitro shoot				2,4-D (1)	809050						
	5 y old plant				2,4-D (5)*							
					2,4-D (1)							
flower bud	field grown plant	Embryogenic callus induction:										Shoyama et al. 1987
		MS	none	sucrose (30)	2,4-D (1)	75	3 mths	nd	71% formed shoots 75% of these rooted		unknown	
flower stalk	*in vitro* plantlet	DIRECT ORGANOGENESIS							71.6% -normal shoots 40.5% -rooted		unknown	Lim et al. 1997
***Panax pseudoginseng* var. *japonicus* (Chikusetsu ginseng)**												
flower bud	2 wk old seedling	Induction of SE and callus:							85% formed shoots after 6 wks		unknown	Fujioka et al. 1986 (as cited in Shoyama et al. 1995)
		MS	none	sucrose (30)	2,4-D (1)	80	15 wks	nd				
		Induction of secondary embryogenesis (10 wks):										
		MS	none	sucrose	NAA (2)* and BA (10)	100	25 wks	nd				
***Panax pseudoginseng* var. *notoginseg* (Sanchi ginseng)**												
flower bud	field grown plant	Embryogenic callus induction:							-85% formed shoots		n/a	Shoyama et al. 1997
		MS	none	sucrose (30)	2,4-D (1)	60	18 wks	nd				
***Panax ginseng* C. A. Meyer (Korean ginseng)**												
root transverse thin cell layers	*in vitro* seedling: (15 d)	MS	none	sucrose (30)	2,4-D (1.105)	0	n/a	n/a	n/a	n/a	n/a	Ahn et al. 1996
root	6 y old roots	Embryogenic callus induction:							n/a	n/a	n/a	Ahn & Dong 1992
		MS	none	sucrose (30)	2,4-D (3)							

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
		SE induction from callus:										
		MS	none	sucrose (50)	2,4-D (1)*	nd	8-9 mths	lots				
root	mature root	Induction of SE and callus:										
		MS	none	sucrose (30)	2,4-D (1)	nd	8 mths	numerous	nd	nd	unknown	Chang & Hsing 1980
root	*in vitro* plantlet	DIRECT ORGANOGENESIS							71.6% -normal shoots 40.5% -rooted		unknown	Lim et al. 1997
root	5 y old plant	Embryogenic callus induction and ORGANOGENESIS				n/a	n/a	n/a	nd	nd	unknown	Furuya et al. 1986
***Panax quinquefolius* L. (North American ginseng)**												
root	3-5 y old field grown plants	Embryogenic callus induction (3 mths):							0	n/a	n/a	Tirajoh et al. 1998
		MS	none	sucrose (30)	dicamba (2)* and K (1.08)*							
		SE induction from callus (3 mths):										
		MS	none	sucrose (30)	dicamba (2)*	15.6	6 mths	nd				
root	4 y old field grown plants	Callus induction (8 wks):							30	nd	unknown	Wang 1990
		MS	none	sucrose (30)	2,4-D(2) + K(1)							
		Embryogenic callus and SE induction (3 wks):										
		MS	none	sucrose (30)	dicamba (2)	nd	nd	nd				
***Panax ginseng* C. A. Meyer (Korean ginseng)**												
flower bud		Embryogenic callus induction:							nd	1 mth	unknown	Kishira et al. 1992

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
	in vitro shoot	MS	none	sucrose (30)	2,4-D (1)	809050	3 mths	nd				
	5 y old plant				2,4-D (5)*							
					2,4-D (1)							
flower bud	field grown plant	Embryogenic callus induction:										Shoyama et al. 1987
		MS	none	sucrose (30)	2,4-D (1)	75	3 mths	nd	71% formed shoots 75% of these rooted		unknown	
flower stalk	*in vitro* plantlet	DIRECT ORGANOGENESIS							71.6% -normal shoots 40.5% -rooted		unknown	Lim et al. 1997
***Panax pseudoginseng* var. *japonicus* (Chikusetsu ginseng)**												
flower bud	2 wk old seedling	Induction of SE and callus:							85% formed shoots after 6 wks		unknown	Fujioka et al. 1986 (as cited in Shoyama et al. 1995)
		MS	none	sucrose (30)	2,4-D (1)	80	15 wks	nd				
		Induction of secondary embryogenesis (10 wks):										
		MS	none	sucrose	NAA (2)* and BA (10)	100	25 wks	nd				
***Panax pseudoginseng* var. *notoginseg* (Sanchi ginseng)**												
flower bud	field grown plant	Embryogenic callus induction:							-85% formed shoots		n/a	Shoyama et al. 1997
		MS	none	sucrose (30)	2,4-D (1)	60	18 wks	nd				
***Panax ginseng* C. A. Meyer (Korean ginseng)**												
multiple shoot derived by organogenesis	5 y old root (see Furuya et al. 1986)	Embryogenic tissue induction (6 wks):							80% (from sucrose (80) treatment)		6 wks	Asaka et al. 1994
		MS	culture 24 hrs at 35°C	sucrose (30)	none							
		SE induction from embryogenic tissue (6 wks):				nd	12 wks					
		MS	none	sucrose: (80) (100)*	none			~80/g ~100/g				

Explant	Source	Somatic Embryo Induction Medium				Regeneration Efficiency			Plantlet Recovery		Field Trial	Reference
		Salts	Modifications	Sugars (g/L)	PGRs (mg/L)	% with SE	time	SE/explant	%	time	% emergence	
multiple shoot derived by organogenesis	5 y old root (see Furuya et al. 1986)	MS	pretreatment[12hr*]: 25°C 35°C 43°C	sucrose (30)	K (1)	109550	6 wks	9.3/g ±6.5 75.7/g ±19.6 25.2/g ±12.1	nd		6 wks	Asaka et al. 1993
***Eleutherococcus senticosus* (Siberian ginseng)**												
hypocotyl	1 wk old seedling	MS	none	sucrose (30)	2,4-D (1)	low	nd	nd		2 ½ mths	unknown	Choi et al. 1999b
		Embryogenic callus induction from SE (2 mths):										
		MS	2X salts	sucrose (30)	2,4-D (1)	n/a	n/a	n/a				
		SE induction from embryogenic callus (1 mth):										
		MS	none	sucrose (30)	none	nd	nd	1570 ±265 per .5g callus	97			
		as above but in cell suspension				nd	nd	5514 ±487 per .2g callus	76			